MiR199a is implicated in embryo implantation by regulating *Grb10* in rat

Hong-Fei Xia^{1,2}, Jing-Li Cao^{1,2}, Xiao-Hua Jin¹ and Xu Ma^{1,2}

¹Reproductive and Genetic Center of National Research Institute for Family Planning, Beijing 100081, China and

²Graduate School, Peking Union Medical College, Beijing 100730, China

Correspondence should be addressed to X Ma; Email: genetic@263.net.cn or to H-F Xia; Email: hongfeixia@126.com

Abstract

MiR199a was found to be differentially expressed in rat uteri between the prereceptive and receptive phase via microRNA (miRNA) microarray analysis in our previous study. However, the role of *miR199a* in rat embryo implantation remained unknown. In the study, northern blot results showed that the expression levels of *miR199a* were higher on gestation days 5 and 6 (g.d.5–6) in rat uteri than on g.d.3–4 and g.d.7–8. *In situ* localization of *miR199a* in rat uteri showed that *miR199a* was mainly localized in the stroma or decidua. The expression of *miR199a* was not significantly different in the uteri of pseudopregnant rats and evidently increased in the uteri of rats subjected to activation of delayed implantation and experimentally induced decidualization. Treatment with 17 β -estradiol or both 17 β -estradiol and progesterone significantly diminished *miR199a* levels. Gain of function of *miR199a* in endometrial stromal cells isolated from rat uteri inhibited cell proliferation and promoted cell apoptosis. Loss of function of *miR199a* displayed opposite roles on cell proliferation and apoptosis. Further investigation uncovered a significant inverse association between the expression of *miR199a* and growth factor receptor-bound protein 10 (*Grb10*), an imprinted gene, and *miR199a* could bind to the 3'UTR of *Grb10* to inhibit *Grb10* translation. In addition, *in vivo* analysis found that the immunostaining of GRB10 was attenuated in the stroma or decidua from g.d.4 to 6, contrary to the enhancement of *miR199a*. Collectively, upregulation of *miR199a* in rat uterus during the receptive phase is regulated by blastocyst activation and uterine decidualization. Enforced *miR199a* expression suppresses cell proliferation partially through targeting *Grb10*.

Reproduction (2014) **147** 91–99

Introduction

Pregnancy begins with the fertilization of the ovum, followed by implantation of the blastocyst into the maternal uterus (Carson *et al.* 2000, Koot & Macklon 2013). Implantation is an essential step in the development of a pregnancy (Koot & Macklon 2013). Blastocysts can implant only if the endometrium has been induced, a transient state of endometrial receptivity for embryo implantation that lasts for <24 h (Quinn *et al.* 2006, Granot *et al.* 2012). Endometrial receptivity for embryo implantation in rats occurs on day 5 of pregnancy. The time surrounding the window of receptivity is referred to as the peri-implantation period. In response to implanting embryos, the underlying endometrial stromal cells (ESCs) undergo decidualization (Zhang *et al.* 2012b). Although a large number of studies have been conducted to explore the exact molecular mechanisms of embryo implantation, there are many unanswered questions about the specific mechanisms associated with the onset of uterine receptivity and embryo implantation.

MicroRNAs (miRNAs) are short noncoding RNAs that are involved in several important biological processes

through the regulation of gene expression at the posttranscriptional level (Pan *et al.* 2013, Wang *et al.* 2013b). The miRNAs elicit their effects by binding to the 3'UTR of their target mRNAs, leading to the inhibition of translation or the degradation of the mRNA, depending on the degree of complementary base pairing (Guo *et al.* 2013, Han *et al.* 2013, Wang *et al.* 2013a). Our previous study found that *miR199a* was differentially expressed in rat uteri between the prereceptive and receptive phase by miRNA microarray analysis. *MiR199a** was spatiotemporally expressed in mouse uterus during embryo implantation (Chakrabarty *et al.* 2007). However, the expression and function of *miR199a* in embryo implantation in rats is still unknown.

In this study, we report the spatiotemporal profiles of *miR199a* in rat uterus during peri-implantation and the effects of pseudopregnancy, artificial decidualization, activation of delayed implantation, and hormone treatment on *miR199a* expression. In addition, we also test the effects of *miR199a* on cell growth and report the identification of growth factor receptor-bound protein 10 (*Grb10*) as a regulatory target of *miR199a*.

Materials and methods

Experimental animals and protocols

Sexually mature, healthy female Sprague–Dawley rats (220–260 g body weight) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Rats were housed in a temperature- and humidity-controlled room with a 12 h light:12 h darkness cycle. All animal procedures were approved by the Institutional Animals Care and Use Committee of the National Research Institute for Family Planning. Rats were caged overnight with fertile males of the same strain. The presence of a vaginal plug or sperm was considered as day 1 of gestation (g.d.1). Uteri were excised from g.d.3 to 8 in rat and fixed with 4% paraformaldehyde (PFA) solution (Sigma–Aldrich) for *in situ* hybridization or frozen in liquid nitrogen for RNA analysis.

Pseudopregnancy was induced by caging adult females with vasectomized males and mating was confirmed by checking for a vaginal plug (day 1 of pseudopregnancy). The uteri were collected from day 3 to day 7 of pseudopregnancy. On day 5 of pseudopregnancy, when the uteri were optimally sensitized for artificial decidualogenic stimuli, 100 µl olive oil (Sigma–Aldrich) was infused into the lumen of one of the uterine horns to induce artificial decidualization. The contralateral uterine horn, which was not infused with oil, served as a control. At day 7 of pseudopregnancy, the rats were killed and the uterine horns were isolated.

To induce delayed implantation, the pregnant rats on g.d.4 were ovariectomized and injected with progesterone (5 mg/rat, s.c.; Sigma–Aldrich) intraperitoneally to maintain delayed implantation from g.d.5 to 7. Then the rats were treated with 17β-estradiol (0.5 µg/rat; Sigma–Aldrich) to terminate delayed implantation. The rats were killed by stunning and cervical dislocation to collect uteri at 24 h after estrogen treatment. The implantation sites were also identified by i.v. injection of Chicago blue solution (Sigma–Aldrich). To confirm that the rats receiving progesterone only were in a state of delayed implantation, uterine flushings were collected from g.d.5 to 7 and examined for the presence of hatched blastocysts.

To test the effects of steroid hormones on *miR199a* expression, treatment with hormones was started 2 weeks after rats had been ovariectomized. The ovariectomized rats were injected with 17β-estradiol (1 µg/rat) or progesterone (10 mg/rat) at intervals of 24 h for 3 days. All steroids were dissolved in olive oil and injected intraperitoneally. The ovariectomized rats were injected with olive oil only (0.1 ml/rat) as controls.

Northern blot analysis

Total RNA was isolated from uterus with TRIzol reagent (Invitrogen). Aliquots of 40 µg of total RNA per sample were subjected to electrophoresis on a 15% urea–PAGE gel and transferred to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech). After being u.v. cross-linked and baked at 50 °C for 30 min, the membrane was prehybridized with hybridization buffer at 42 °C for 4 h and then hybridized with ³²P-labeled *miR199a* probe (GAACAGGTAGTCTGAACACTGGG) or U6 probe (CGTCCA-ATTTAGTATATGTGCTGCCGAAGCGA) at 40 °C overnight.

Membranes were washed and exposed to PhosphorImager screens (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The bands were analyzed using Quantity One software (Bio-Rad). All experiments were repeated at least three times.

In situ hybridization

Sections of uterus (5 µm) were treated with proteinase K (20 g/ml) for 15 min and refixed in 4% PFA for 15 min. After acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, sections were prehybridized with hybridization buffer (Roche) at 40 °C for 2 h and then hybridized with digoxigenin (DIG)-labeled LNA-*miR199a* probe (LNA-*miR199a* sequence: 5'-DIG-gAacagGtAgTctGaA-cacTggg-3') at 40 °C overnight. The sections were then incubated in buffer containing anti-DIG-antibody for 2 h at 37 °C, followed by staining with 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium chloride (Promega). The sections were hybridized with a DIG-labeled LNA-scrambled probe (LNA-scrambled sequences: 5'-caTtaAtgTcGgaCaaCt-cAat-3') as a negative control. The sections were observed with an Eclipse 80i microscope (Nikon, Tokyo, Japan).

Cell viability assay

In vitro cell viability was estimated with an MTS assay. Briefly, ESCs isolated from rat uteri (5000 cells/well) were seeded in 96-well plates (Costar, Corning, Inc., Corning, NY, USA) in DMEM/F12 culture, and allowed to attach overnight. The cells were then transfected with *miR199a* mimics, premiR control, *miR199a* inhibitor, and anti*miR* control respectively. *miR199a* mimics are chemically synthesized, double-stranded RNAs that mimic mature endogenous *miR199a* after transfection into cells. *miR199a* inhibitors are chemically synthesized, single-stranded, modified RNAs that specifically inhibit endogenous *miR199a* function after transfection into cells. Twenty microliters MTT (5 mg/ml; Sigma–Aldrich) were added to each well 48 h after transfection, and incubated for 4 h. Media were then removed and 150 µl dimethyl sulfoxide (Sigma–Aldrich) was added in each well. Absorbance was recorded at A570 nm with a 96-well plate reader (Bio-Rad 3550). In order to avoid the error related to the absorbance of the replicated wells, each treatment group was replicated in three wells. In order to decrease the experimental error and calculate the s.d. for each group, the experiment was repeated for three times. In every experiment, the mean absorbance of the replicate wells was calculated and then normalized with the mean absorbance of the control. The results were described as a ratio of A570 nm with *miR199a* mimics vs premiR control or *miR199a* inhibitor vs anti*miR* control.

Flow cytometry analysis

Cell apoptosis was performed with flow cytometry analysis by Annexin V-FLUOS staining Kit (Roche), containing 2.5 µl annexin V-FITC stock and 5 µl 20 µg/ml phosphatidylinositol (PI) to determine the phosphatidylserine exposure on the outer plasma membrane. After incubation for 15 min at room temperature in a light-protected area, the specimens were quantified by flow cytometry (BD Biosciences, San Jose,

CA, USA), acquiring 8000 events. In order to avoid the experimental error within groups and ensure data consistency in each group, each treatment was repeated twice within an experiment. In order to decrease the experimental error between groups and calculate the s.d. for each group, the experiment was repeated three times.

Construction of luciferase reporter plasmids and dual luciferase activity assay

The *Grb10* 3'UTR sequences were amplified by PCR from human genomic DNA using the primers as follow: forward/ SpeI: GGCCTAGTCCTCTTGGCCACTCAGGCT; reverse/ Apal: CATGGGCCCATGGGAAGGACTGGGGTGTC. After being double digested with SpeI and Apal, the PCR product was cloned into the downstream of firefly luciferase gene in pGL3 control vector (Promega). Deleting *miR199a* target sites in the 3'UTR of *Grb10* was used as negative control. All these constructs were verified by DNA sequencing.

For luciferase reporter assays, HEC-1B cells were seeded in 48-well plates and allowed to attach overnight, then transfected with 50 nM of *miR199a* mimics, *miR199a* inhibitor, premiR control, or anti-miR control (GenePharma Co., Ltd, Shanghai, China) by using lipofectamine 2000 (Invitrogen). HEC-1B cells were also transfected with wild or mutant reporter plasmid. PRL-TK containing Renilla luciferase was co-transfected for data normalization. Two days later, cells were harvested and the luciferase activity was measured with the dual luciferase assay system (Promega). Each treatment was performed in triplicate in three independent experiments. Firefly luciferase activity was normalized to Renilla luciferase activity and the results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Western blot

Protein lysates from HEC-1B cells were boiled in SDS/β-mercaptoethanol sample buffer, and 60 μg samples were loaded into each lane of 12% polyacrylamide gels. The proteins were separated by electrophoresis and transferred to polyvinylidene fluoride membrane (PVDF; Amersham Pharmacia Biotech) by electrophoretic transfer. The membrane was incubated with rabbit anti-GRB10 (Proteintech Group, Inc., Chicago, IL, USA) or β-ACTIN polyclonal antibody (Santa Cruz Biotechnology, Inc.) overnight at 4 °C. The specific protein-antibody complex was detected by using HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Detection by the chemiluminescence reaction was executed using the ECL kit (Millipore, Billerica, MA, USA). β-ACTIN was used as a loading control. The experiment was repeated at least three times. The bands were analyzed using Quantity One analyzing system (Bio-Rad).

Immunohistochemistry

Sections (4 μm) of uterine tissues were deparaffinized in xylene and rehydrated in descending ethanol series. Antigen retrieval was accomplished through microwave irradiation of the sections in 10 mM sodium citrate buffer. Slides were incubated

with rabbit anti-GRB10 polyclonal antibody (Proteintech Group, Inc.), then incubated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The antibody stains were developed by the addition of diaminobenzidine (DAB; Sigma-Aldrich) and the nuclei were stained with hematoxylin (Sigma-Aldrich). The sections were incubated with normal goat serum as negative control. Samples were viewed under Nikon TE 2000-U microscope (Nikon).

Statistical analysis

There were at least three rats in each treatment group. The results of northern blot and *in situ* hybridization were repeated three times. All values were reported as the mean ± S.E.M. Statistical analysis was done using one-way ANOVA. When significant effects of treatments were indicated, the Student-Newman-Keuls multi-range test was applied using SPSS version 13.0 (SPSS, Inc.). $P < 0.05$ was considered statistically significant.

Results

MiR199a expression and regulation in rat uterus during early pregnancy

In this study, we first examined the expression pattern of *miR199a* in rat uterus during the peri-implantation period by northern blot. The expression levels of

Figure 1 The dynamics of *miR199a* expression in rat uterus during early pregnancy. The expression of *miR199a* in rat uteri from day 3 to day 8 of pregnancy was detected by northern blot. Hybridization was conducted with a ^{32}P -labeled probe specific for *miR199a* or *U6*. *U6* snRNA was used as a loading control to normalize for gel loading and transfer. The histogram represents the *miR199a* levels quantified by densitometric analysis and expressed as a ratio of *miR199a* to *U6* intensity. * $P < 0.05$.

Figure 2 The expression pattern of *miR199a* in rat uteri in the model of pseudopregnancy, delayed implantation, and artificial decidualization. The expression of *miR199a* in rat uteri in the model of pseudopregnancy (A), delayed implantation (B), and artificial decidualization (C) was detected by northern blot. Hybridization was conducted with a 32 P-labeled probe specific for *miR199a* or *U6*. *U6* snRNA was used as a loading control to normalize for gel loading and transfer. The histogram represents the *miR199a* levels quantified by densitometric analysis and expressed as a ratio of *miR199a* to *U6* intensity. * $P < 0.05$, ** $P < 0.01$.

miR199a on g.d.5–6 were higher than on g.d.3–4 and g.d.7–8 ($P < 0.05$; Fig. 1).

To understand whether *miR199a* expression was dependent upon the presence of embryos, the expression of *miR199a* in uteri from day 3 to day 7 of pseudopregnancy was detected by northern blot (Fig. 2A). Low levels of *miR199a* were detected in the uteri on day 3 of pseudopregnancy. The expression levels of *miR199a* in the uteri from day 4 to day 7 of pseudopregnancy were similar to those on day 3 of pseudopregnancy, implying that *miR199a* expression was not dependent upon the presence of embryos.

To detect whether *miR199a* expression was dependent upon embryo status, activation of a delayed implantation model was used for northern blot analysis. The ovariectomized pregnant rats were injected with progesterone to maintain delayed implantation. The delayed-implantation rats were treated with 17β -estradiol to terminate delayed implantation and activate the blastocysts. Low levels of *miR199a* were found in the uterus under the delayed implantation condition, but the *miR199a* levels were significantly enhanced after implantation was activated with estrogen treatment ($P < 0.05$; Fig. 2B), suggesting that *miR199a* expression was dependent upon activated blastocysts, not upon dormant blastocysts.

To test whether *miR199a* expression was regulated by decidualization, a model of experimentally induced decidualization was used for northern blot analysis. The expression levels of *miR199a* in the decidualized uterus

were higher than those in the nonstimulated uterus on day 7 of pseudopregnancy ($P < 0.01$; Fig. 2C).

The effects of steroid hormones on *miR199a* expression

The effects of steroid hormones on *miR199a* expression were examined by northern blot (Fig. 3) analysis. Low levels of *miR199a* were detected in the ovariectomized rat uterus. Progesterone treatment slightly increased the expression of *miR199a*. Treatment with 17β -estradiol significantly boosted *miR199a* expression ($P < 0.05$). Treatment with 17β -estradiol and progesterone further enhanced *miR199a* levels ($P < 0.01$).

Localization of *miR199a* in rat uteri

Localization of *miR199a* in rat uterus during early pregnancy was detected by *in situ* hybridization. *miR199a* was mainly located in the superficial and deep stroma and the circular and longitudinal smooth muscle on g.d.4 (Fig. 4A). On g.d.5 and g.d.6, positive signals of *miR199a* were strengthened in the superficial and deep stroma or the primary and secondary decidual zone (Fig. 4B and C). In the model of delayed implantation, weak *miR199a* staining was found in the superficial stroma under delayed implantation conditions (Fig. 4D and E). After delayed implantation was terminated by estrogen treatment and embryos began to implant, strong *miR199a* signals were detected in the

Figure 3 The effects of steroid hormones on *miR199a* expression in rat uterus. The effects of steroid hormones on *miR199a* expression in uteri of ovariectomized rats were detected by northern blot. Hybridization was done with a 32 P-labeled probe for *miR199a* or *U6*. *U6* snRNA was used as a loading control to normalize for gel loading and transfer. The histogram represents the *miR199a* levels quantified by densitometric analysis and expressed as a ratio of *miR199a* to *U6* intensity. * $P < 0.05$, ** $P < 0.01$.

superficial stroma (Fig. 4F). In the model of artificial decidualization, weak staining was found in the superficial and deep stroma in the control uterine horn on day 7 of pseudopregnancy (Fig. 4G). However, in the oil-infused uterus, strong *miR199a* signals were found in the primary and secondary decidual zone and myometrium (Fig. 4H).

The effects of *miR199a* on cell proliferation

The effects of *miR199a* on cell proliferation were detected by MTS assay. In order to detect the alteration of cell proliferation, ESCs were transfected with *miR199a* mimics or inhibitor (Fig. 5A). The relative proliferation rates in ESCs transfected with *miR199a* mimics were significantly diminished compared with premiR control ($P < 0.05$). The relative proliferation rates in cells transfected with *miR199a* inhibitor were obviously augmented compared with anti-miR control ($P < 0.05$). These results show that overexpression of *miR199a* inhibits cell proliferation, while low expression of *miR199a* promotes cell viability.

The effects of *miR199a* on cell apoptosis

To further explore the role of *miR199a* in controlling cell growth, apoptosis in ESCs was determined by flow cytometry analysis (Fig. 5B). Compared with premiR control, transfection with *miR199a* mimics enhanced the number of early (7.37 vs 1.47%) and late apoptotic cells (11.30 vs 3.14%). When cells were transfected with *miR199a* inhibitor, the number of early apoptotic cells was slightly reduced (1.75 vs 2.71%), and the number of late apoptotic cells was observably lessened compared with anti-miR control (2.10 vs 4.64%).

Prediction and confirmation of the target gene of *miR199a*

To analyze the molecular mechanisms in which *miR199a* was implicated, we explored its target gene. An online search of *miR199a* targets by miRanda (<http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl>)

Figure 4 *In situ* localization of *miR199a* in rat uterus. Uteri sections from day 4 (A), day 5 (B), day 6 (C) of pregnancy, delayed implantation (D and E) and activation of delayed implantation (F), and non-stimulated (G) and stimulated (H) artificial decidualization were subjected to *in situ* hybridization using a DIG-labeled LNA probe specific to *miR199a*. To evaluate the specificity of the probe, negative control staining was performed by substituting a DIG-labeled LNA-scrambled probe for the DIG-labeled LNA-*miR199a* probe (I). Blue staining indicates hybridization signals. The photographs are shown at $\times 100$ original magnification. l, uterine lumen; le, luminal epithelium; mg, maternal glands; ge, glandular epithelium; ss, superficial stroma; ds, deep stroma; bv, blood vessels; m, myometrium; cm, circular smooth muscle; lm, longitudinal smooth muscle; pdz, primary decidual zone; sdz, secondary decidual zone.

Figure 5 The effects of *miR199a* on proliferation and apoptosis of ESCs. ESCs were transfected with premiR control, *miR199a* mimics, anti-miR control, or *miR199a* inhibitor respectively. After 48 h of transfection, cell proliferation was determined by MTS assay (A). All experiments were performed at least three times and calculated cell proliferation as stimulation index (SI) (ratio of A570 nm with *miR199a* mimics vs premiR control or *miR199a* inhibitor vs anti-miR control). Cell apoptosis was determined by flow cytometry (B). The average percentage of apoptosis cells was analyzed in cells transfected by *miR199a* mimics and inhibitor at early and late stages. Lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, late apoptosis/necrosis cells (annexin V-FITC and PI positive). The average percentage of early and late apoptotic cells (representatives of three separate experiments) was shown in the lower right and upper right panels respectively. * $P < 0.05$.

TargetScan databases (<http://genes.mit.edu/targetscan.test/ucsc.html>), and PicTar (<http://pictar.mdc-berlin.de/>) showed that *Grb10* was a putative mRNA target of *miR199a*. TargetScan, miRanda, and PicTar predictions showed that there was one conservative *miR199a*-responsive element in the 3'UTR of *Grb10* (Fig. 6A).

To validate *Grb10* as the target of *miR199a*, we set up a luciferase reporter assay. The 3'UTR fragment of wt-*Grb10* of rat containing the binding sites of *miR199a* was cloned downstream of the firefly luciferase (designated as wt-Grb10) for the dual luciferase assay (Fig. 6B). Wt-Grb10s were co-transfected with *miR199a* mimics or inhibitor. Compared with premiR control, the luciferase activity in HEC-1B cells co-transfected with *miR199a* mimics and wt-Grb10 was decreased by

about 43.19% ($P < 0.01$). Furthermore, the luciferase activity was upregulated by about 33.43% in cells co-transfected with *miR199a* inhibitor and wt-Grb10 compared with anti-miR control ($P < 0.01$). These results indicate that *miR199a* dysregulation affects the binding of *miR199a* and the 3'UTR of *Grb10*, leading to the alteration in *Grb10* translation.

Base deletion of the seed region was also performed to further confirm the binding site for *miR199a* in the 3'UTR of *Grb10* (Fig. 6C). Deletion of the putative *miR199a* binding region in the 3'UTR of *Grb10* (designated as del-Grb10) was used as control. The luciferase activity was decreased by 80.66% in the cells co-transfected with *miR199a* mimics and wt-Grb10 compared with del-Grb10 ($P < 0.01$). These data indicate that *miR199a* may restrain target gene expression through binding to the seed sequence in the 3'UTR of *Grb10*.

MiR199a regulates endogenous GRB10 expression

To ascertain the effects of *miR199a* on endogenous GRB10 expression, we transfected the HEC-1B cells with *miR199a* mimics or inhibitor (Fig. 7). Compared with premiR control, GRB10 protein levels were significantly decreased by *miR199a* mimics ($P < 0.05$). In addition, compared with anti-miR control, GRB10 protein levels in the HEC-1B cells were measurably increased by *miR199a* inhibitor ($P < 0.05$).

The expression of GRB10 in rat uterus

To further explore the expression profiles of *miR199a* target gene, *Grb10*, *in vivo*, the expression of *Grb10* in rat uteri from day 4 to day 6 of pregnancy was detected by immunohistochemistry. On day 4 of pregnancy, strong immunostaining of GRB10 was found in the luminal and glandular epithelium, the superficial stroma, and the myometrium. Moderate intense immunostaining was found in the deep stroma (Fig. 8A). On day 5 of pregnancy, moderate positive hybridization signals were detected in the luminal and glandular epithelium and the myometrium. Weak immunoreaction was found in the stroma (Fig. 8B). On day 6 of pregnancy, moderate immunoreaction was restricted to the luminal and glandular epithelium. Slight immunoreaction was present in the primary and secondary decidual zone and the myometrium (Fig. 8C). These results indicate that the immunostaining of GRB10 in rat uterus on day 4 of pregnancy is stronger than that on day 5 and 6 of pregnancy.

Discussion

Chakrabarty *et al.* (2007) reported that *miR199a** was differentially expressed in the mouse uterus during

Figure 6 The prediction and confirmation of the *miR199a* target gene. *miR199a*-binding sites in the 3'UTR of *Grb10* were compared in cross-species (A). The 3'UTR fragment of wt-*Grb10* of rat containing the binding sites of *miR199a* was cloned into the downstream of firefly luciferase reporter gene in pGL3 control vector (designated as wt-*Grb10*) for the dual luciferase assay (B). HEC-1B cells were co-transfected with *miR199a* mimics, premiR control, *miR199a* inhibitor, or anti-miR control, and wt-*Grb10* for dual luciferase assay. Deleting the putative *miR199a* binding region in the 3'UTR of *Grb10* (designated as del-*Grb10*) was used as control. HEC-1B cells were co-transfected with *miR199a* and wt- or del-*Grb10* for the dual luciferase assay (C). pRL-TK containing Renilla luciferase was co-transfected for data normalization. ** $P < 0.01$.

implantation. However, the expression pattern and function of *miR199a* during embryo implantation in rats remained unknown. To study the role of *miR199a* in embryo implantation, we first examined its spatiotemporal distribution in the rat uterus during the peri-implantation period. The expression of uterine *miR199a* on g.d.5–6 was higher than that on g.d.3–4 and g.d.7–8, and it was mainly located in the stroma or decidua. Embryo transfer experiments have revealed that blastocysts transferred to the uterine lumen of day 4.5 of pseudopregnant recipients remain in a state of dormancy until the endometrium becomes receptive on day 5, then blastocysts are activated and embryo implantation can be initiated (Psychoyos 1973, Kennedy *et al.* 2007).

The spatiotemporal distribution of *miR199a* during early pregnancy suggests that the state of endometrial receptivity and blastocyst activation may promote the expression of *miR199a*.

To further search whether *miR199a* expression was related to the induction of implantation, we measured the effects of pseudopregnancy, delayed implantation, and artificial decidualization on *miR199a* expression. The results showed that *miR199a* levels were similar in rat uteri from day 3 to day 7 of pseudopregnancy. In the model of activation of delayed implantation, the expression of *miR199a* was low under delayed implantation conditions, but it was significantly increased after activation of delayed implantation with estrogen treatment. In the model of artificial decidualization, the expression levels of *miR199a* in the decidualized uterus were significantly enhanced. These results indicate that the increase in *miR199a* expression relies on activated blastocysts and decidualization, which is coincident with the speculation elicited by the expression pattern of *miR199a* in early pregnancy.

Progesterone and estrogen are needed for implantation in rats and mice (Rockwell *et al.* 2002, Chen *et al.* 2003). Progesterone can help to prepare the uterus for implantation and development of a fertilized egg (Aitken 1978). Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation (Ma *et al.* 2003). In this study, we found that *miR199a* expression was significantly upregulated by 17 β -estradiol or both 17 β -estradiol and progesterone treatment in the ovariectomized rat uterus. The gene expression patterns and hormonal levels were different

Figure 7 The effects of *miR199a* on endogenous GRB10 expression. GRB10 protein levels in *miR199a* mimics (A) and inhibitor (B)-treated HEC-1B cells were detected by western blot. Total protein was separated by SDS-PAGE and then transferred to PVDF membrane. The membrane was incubated with GRB10 or B-ACTIN antibody and detected by the ECL detection system. The histogram represents the optical densities of the signals quantified by densitometric analysis and represented as GRB10 intensity/B-ACTIN intensity to normalize for gel loading and transfer. * $P < 0.05$.

Figure 8 The expression of GRB10 in rat uterus. Uteri sections from day 4 (A), day 5 (B), and day 6 (C) of pregnancy were subjected to immunohistochemistry using GRB10 antibody. The sections were incubated with normal goat serum as negative control (D). The staining was developed with DAB and the nuclei were stained with hematoxylin. Brown staining indicates positive immunoreaction. The photographs were shown at $\times 100$ original magnification. l, uterine lumen; le, luminal epithelium; mg, maternal glands; ge, glandular epithelium; ss, superficial stroma; ds, deep stroma; bv, blood vessels; m, myometrium; cm, circular smooth muscle; lm, longitudinal smooth muscle; pdz, primary decidual zone; sdz, secondary decidual zone.

between pregnant and nonpregnant rat uterus (Michie & Head 1994, Shynlova *et al.* 2007). The expression of *miR199a* in the uteri shows no comparison between the pregnant rats and the ovariectomized and steroid-treated nonpregnant rats. However, these results indirectly imply that the effects of progesterone and 17β -estradiol on *miR199a* expression may be one of reasons that *miR199a* expression is upregulated during the embryo implantation period.

To explore the possible role of *miR199a* in rat embryo implantation, we tested the effects of *miR199a* on the growth of ESCs. Gain of function of *miR199a* in ESCs isolated from rat uteri inhibited cell proliferation and promoted cell apoptosis. Loss of function of *miR199a* accelerated cell proliferation and suppressed cell apoptosis. The phenomenon was consistent with the reports of Cheng *et al.* (2012) and Zhang *et al.* (2013a) that *miR199a* suppressed the proliferation of cancer-initiating cells and induced the apoptosis of hepatocellular carcinoma cells in human. Therefore, upregulation of *miR199a* in rat uteri during the implantation phase may contribute to prevention of the excessive invasion of trophoblastic cells.

To study the possible molecular mechanisms by which *miR199a* affected cell growth, we analyzed its target gene. An online search of *miR199a* targets by miRanda, TargetScan, and PicTar showed that there was one conservative *miR199a*-responsive element in the 3'UTR of *Grb10*, an imprinted gene. When *miR199a*

mimics or inhibitor were co-transfected with the recombinant vector bearing the putative *miR199a*-binding sites in the 3'UTR of *Grb10*, luciferase activity was decreased by *miR199a* mimics and increased by *miR199a* inhibitor, suggesting that *Grb10* should be the target gene of *miR199a*. A deletion experiment further confirmed that the binding sites in the 3'UTR of *Grb10* were specific for *miR199a*. In addition, overexpression of *miR199a* reduced the protein levels of GRB10 and knockdown of *miR199a* promoted the expression of GRB10. These data indicate that *Grb10* is not only the target gene of *miR199a*, but the endogenous GRB10 protein levels are also regulated by *miR199a*. Disruption of *Grb10* in the pancreas enhanced B-cell proliferation and protected mice from streptozotocin-induced B-cell apoptosis (Zhang *et al.* 2012a). *Grb10* could interact with the dynein-binding domain (DBD) of Bim and inhibited apoptosis triggered by overexpression of DBD-containing Bim isoforms (Hu *et al.* 2010). Therefore, downregulation of *Grb10* by the high levels of *miR199a* may contribute to promotion of cell proliferation and facilitation of endometrial development.

Although *miR199a* can regulate GRB10 expression in cell lines, it was unknown whether there was the same condition in the rat uterus. The expression of GRB10 in uteri from day 4 to day 6 of pregnant rats was detected by immunohistochemistry in order to validate the above-mentioned results *in vivo*. Strong immunostaining of GRB10 was found in the stroma on day 4 of pregnancy and weak immunostaining of GRB10 was detected in the stroma or decidua on day 5 and 6 of pregnancy. However, weak staining of *miR199a* was observed in the stroma on day 4 of pregnancy. On day 5 and 6 of pregnancy, positive signals of *miR199a* were strengthened in the stroma or decidua. These results show that there is a inverse correlation between the expression of *miR199a* and GRB10 in the rat uterus.

In conclusion, *miR199a* expression was increased in rat uterus during the receptive phase and regulated by blastocyst activation and uterine decidualization. Enforced *miR199a* expression promoted ESC apoptosis, which may be partially through downregulation of *Grb10*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by grants from Natural Science Foundation of China (No. 81370720) and National Research Institute for Family Planning (No. 2012GJSSJKA03), and International S&T Cooperation Program of China (2012DFB30130).

References

- Aitken RJ 1978 The hormonal control of implantation. *Ciba Foundation Symposia* **64** 53–83.
- Alexander MS, Kawahara G, Motohashi N, Casar JC, Eisenberg I, Myers JA, Gasperini MJ, Estrella EA, Kho AT, Mitsuhashi S *et al.* 2013 MicroRNA-199a is induced in dystrophic muscle and affects WNT signaling, cell proliferation, and myogenic differentiation. *Cell Death and Differentiation* **20** 1194–1208. (doi:10.1038/cdd.2013.62)
- Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA & Yoshinaga K 2000 Embryo implantation. *Developmental Biology* **223** 217–237. (doi:10.1006/dbio.2000.9767)
- Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H & Dey SK 2007 MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *PNAS* **104** 15144–15149. (doi:10.1073/pnas.0705917104)
- Chen B, Zhang D & Pollard JW 2003 Progesterone regulation of the mammalian ortholog of methylcitrate dehydratase (immune response gene 1) in the uterine epithelium during implantation through the protein kinase C pathway. *Molecular Endocrinology* **17** 2340–2354. (doi:10.1210/me.2003-0207)
- Cheng W, Liu T, Wan X, Gao Y & Wang H 2012 MicroRNA-199a targets CD44 to suppress the tumorigenicity and multidrug resistance of ovarian cancer-initiating cells. *FEBS Journal* **279** 2047–2059. (doi:10.1111/j.1742-4658.2012.08589.x)
- Granot I, Gnainsky Y & Dekel N 2012 Endometrial inflammation and effect on implantation improvement and pregnancy outcome. *Reproduction* **144** 661–668. (doi:10.1530/REP-12-0217)
- Guo Y, Ying L, Tian Y, Yang P, Zhu Y, Wang Z, Qiu F & Lin J 2013 miR-144 downregulation increases bladder cancer cell proliferation by targeting EZH2 and regulating Wnt signaling. *FEBS Journal* **280** 4531–4538. (doi:10.1111/febs.12417)
- Han ZB, Yang Z, Chi Y, Zhang L, Wang Y, Ji Y, Wang J, Zhao H & Han ZC 2013 MicroRNA-124 suppresses breast cancer cell growth and motility by targeting CD151. *Cellular Physiology and Biochemistry* **31** 823–832. (doi:10.1159/000350100)
- Hu ZQ, Zhang JY, Ji CN, Xie Y, Chen JZ & Mao YM 2010 Grb10 interacts with Bim L and inhibits apoptosis. *Molecular Biology Reports* **37** 3547–3552. (doi:10.1007/s11033-010-0002-9)
- Kennedy TG, Gillio-Meina C & Phang SH 2007 Prostaglandins and the initiation of blastocyst implantation and decidualization. *Reproduction* **134** 635–643. (doi:10.1530/REP-07-0328)
- Koot YE & Macklon NS 2013 Embryo implantation: biology, evaluation, and enhancement. *Current Opinion in Obstetrics & Gynecology* **25** 274–279. (doi:10.1097/GCO.0b013e3283630d94)
- Ma WG, Song H, Das SK, Paria BC & Dey SK 2003 Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *PNAS* **100** 2963–2968. (doi:10.1073/pnas.0530162100)
- Michie HJ & Head JR 1994 Tenascin in pregnant and non-pregnant rat uterus: unique spatio-temporal expression during decidualization. *Biology of Reproduction* **50** 1277–1286. (doi:10.1095/biolreprod50.6.1277)
- Pan X, Wang R & Wang ZX 2013 The potential role of miR-451 in cancer diagnosis, prognosis, and therapy. *Molecular Cancer Therapeutics* **12** 1153–1162. (doi:10.1158/1535-7163.MCT-12-0802)
- Psychoyos A 1973 Hormonal control of oviimplantation. *Vitamins and Hormones* **31** 201–256.
- Quinn CE, Simmons DG & Kennedy TG 2006 Expression of cystatin C in the rat endometrium during the peri-implantation period. *Biochemical and Biophysical Research Communications* **349** 236–244. (doi:10.1016/j.bbrc.2006.08.054)
- Rockwell LC, Pillai S, Olson CE & Koos RD 2002 Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. *Biology of Reproduction* **67** 1804–1810. (doi:10.1095/biolreprod.102.006700)
- Shynlova O, Tsui P, Dorogin A, Langille BL & Lye SJ 2007 The expression of transforming growth factor β in pregnant rat myometrium is hormone and stretch dependent. *Reproduction* **134** 503–511. (doi:10.1530/REP-07-0004)
- Wang K, Liu F, Zhou LY, Ding SL, Long B, Liu CY, Sun T, Fan YY, Sun L & Li PF 2013a miR-874 regulates myocardial necrosis by targeting caspase-8. *Cell Death & Disease* **4** e709. (doi:10.1038/cddis.2013.233)
- Wang X, Zhao Y, Zhang X, Badie H, Zhou Y, Mu Y, Loo LS, Cai L, Thompson RC, Yang B *et al.* 2013b Loss of sorting nexin 27 contributes to excitatory synaptic dysfunction by modulating glutamate receptor recycling in Down's syndrome. *Nature Medicine* **19** 473–480. (doi:10.1038/nm.3117)
- Zhang J, Zhang N, Liu M, Li X, Zhou L, Huang W, Xu Z, Liu J, Musi N, DeFronzo RA *et al.* 2012a Disruption of growth factor receptor-binding protein 10 in the pancreas enhances β -cell proliferation and protects mice from streptozotocin-induced β -cell apoptosis. *Diabetes* **61** 3189–3198. (doi:10.2337/db12-0249)
- Zhang L, Patterson AL, Zhang L, Teixeira JM & Pru JK 2012b Endometrial stromal β -catenin is required for steroid-dependent mesenchymal–epithelial cross talk and decidualization. *Reproductive Biology and Endocrinology* **10** 75. (doi:10.1186/1477-7827-10-75)
- Zhang J, Wu GQ, Zhang Y, Feng ZY & Zhu SM 2013a Propofol induces apoptosis of hepatocellular carcinoma cells by upregulation of microRNA-199a expression. *Cell Biology International* **37** 227–232. (doi:10.1002/cbin.10034)
- Zhang S, Liu L, Wang R, Tuo H, Guo Y, Yi L, Wang J & Wang D 2013b MiR199a 5p promotes migration and tube formation of human cytomegalovirus-infected endothelial cells through downregulation of SIRT1 and eNOS. *Archives of Virology*.

Received 7 July 2013

First decision 29 July 2013

Revised manuscript received 11 October 2013

Accepted 22 October 2013